(For Laboratory Use Only)
ATS Labs Project # A 10858 -
LC 1/28/11

ATS LABS

PROTOCOL

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Virus: Murine Norovirus (MNV-1)

PROTOCOL NUMBER

PHL01121610.MNV

PREPARED FOR

Pharmacal Research Laboratories Inc.
562 Captain Neville Drive
Waterbury, CT 06705

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PREPARED BY

Mary Miller, M.T.
Research Scientist II

DATE

December 16, 2010

EXACT COPY
INITIALS JL DATE 3/8/11

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Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

SPONSOR: Pharmacal Research Laboratories Inc.
562 Captain Neville Drive
Waterbury, CT 06705

TEST FACILITY: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PURPOSE

The purpose of this study is to evaluate the virucidal efficacy of a test substance against Murine Norovirus according to test criteria and methods approved by the U.S. Environmental Protection Agency and/or the Sponsor chosen regulatory agency for registration of a product as a virucide. See the "Study Information" page for the regulatory agency or agencies chosen by the Sponsor.

TEST SUBSTANCE CHARACTERIZATION

Test substance characterization as to content, stability, solubility, storage, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor. The test substance shall be characterized before the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is January 4, 2011. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of January 25, 2011. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, because of failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs nor any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the chosen regulatory agency of its submission concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

The U.S. Environmental Protection Agency (EPA) and other regulatory agencies require that a specific virucidal claim for a disinfectant intended for use on hard surfaces be supported by appropriate scientific data demonstrating the efficacy of the test substance against the claimed virus. The agency will accept adequate data generated by any appropriate technique in support of a virucidal efficacy claim. This is accomplished by treating the target virus with the disinfectant (test substance) under conditions, which simulate as closely as possible, in the laboratory, the actual conditions under which the disinfectant is designed to be used. For disinfectant products intended for use on hard surfaces (dry, inanimate environmental surfaces), a carrier method is used in the generation of the supporting virological data. The RAW 264.7 macrophage cell line, which supports the growth of the Murine Norovirus, will be used in this study. The experimental design in this protocol meets these requirements.

TEST PRINCIPLE

A film of virus, dried on a glass surface, is exposed to the test substance for a specified exposure time. Following exposure, the virucidal and cytotoxic activities are removed from the virus-test substance mixture, and the mixture is assayed for viral infectivity by an accepted assay method. Appropriate virus, test substance cytotoxicity, and neutralization controls are run concurrently.

STUDY DESIGN

The appropriate number of dried virus films will be prepared in parallel and used as follows:

One film for each batch of test substance assayed, per exposure time requested, for submission to the EPA or Australian Therapeutic Goods Administration (TGA) and five films for each batch of test substance assayed, per exposure time requested, for submission to Health Canada.

One film for virus control titration (titer of virus after drying), per exposure time requested, for submission to the EPA or Australian Therapeutic Goods Administration (TGA) and five films, per exposure time requested, for submission to Health Canada.

Following the specified exposure time, resuspended virus-test substance mixtures will be detoxified and made non-virucidal by immediately adding the contents to a Sephadex gel filtration column followed by 10-fold serial dilutions in test medium. Each dilution is inoculated into indicator cell cultures. Each dilution is titrated in indicator cell cultures using four cultures for each dilution. The resuspended virus control film and each batch of test substance alone will be treated in exactly the same manner. For analysis of infectivity, cultures will be held for the appropriate incubation period at the end of which time cultures will be scored for the presence of the test virus. Cultures will be monitored at that time for cell viability. Uninfected indicator cell cultures will be carried in parallel and similarly monitored. For analysis of cytotoxicity, the viability of cultures inoculated with dilutions of each test and control will be determined. In addition to the above titrations for infectivity and cytotoxicity, the residual virucidal activity of the test substance after neutralization will be determined by adding a low titer of stock virus to each dilution of the test substance (cytotoxicity control dilutions). The resulting mixtures of dilutions are assayed for infectivity in order to determine the dilution(s) of test substance at which virucidal activity, if any, is retained.

VIRUS

The MNV-1.CW1 strain of Murine Norovirus (MNV-1) to be used for this study was obtained from Washington University, St. Louis, MO. Stock virus is prepared by collecting the supernatant culture fluid from infected culture cells. The cells are disrupted and cell debris removed by centrifugation. The supernatant is removed, aliquoted, and the high titer stock virus may be stored at ≤-70°C until the day of use. On the day of use, an aliquot is removed, thawed, and maintained at a refrigerated temperature until used in the assay. **Note:** If the Sponsor requests an organic soil load challenge, fetal bovine serum (FBS) will be incorporated into the stock virus aliquot. The percent FBS contained in the stock virus aliquot will be adjusted to yield the percent soil load requested.

INDICATOR CELL LINE

RAW 264.7 cells, a continuous mouse macrophage cell line originally obtained from Washington University, St. Louis, MO, are propagated by ATS Labs personnel and used as the indicator cell line for this assay. The cells are seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2. The confluency of the cells will be appropriate for the test virus. Alternatively, cells received from the American Type Culture Collection (ATCC), Manassas, VA may be used. The source of the cells will be documented in the raw data and reported.

All cell culture documentation is retained for the cell cultures used in this assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

TEST MEDIUM

The test medium used for this assay is Complete 2X MEM.

PREPARATION OF TEST SUBSTANCE

The dilution of test substance(s) will be used as recommended by the Sponsor. The product will be pre-equilibrated to the desired test temperature if applicable. Two batches of test substance must be assayed for registration of a test substance as a virucide with the EPA and TGA. Three batches of test substance must be assayed for registration of a test substance as a virucide with Health Canada if a Poliovirus type 1 claim has not been made and two batches are acceptable if a Poliovirus type 1 claim has been made.

PREPARATION OF VIRUS FILMS

Films of virus will be prepared by spreading 0.2 mL of virus inoculum uniformly over the bottom of the appropriate number of 100 X 15mm sterile glass petri dishes. The virus will be air-dried at 10°C-30°C until visibly dry (≥ 20 minutes). The drying conditions (temperature and humidity) will be appropriate for the test virus for the purpose of obtaining maximum survival following drying. The actual drying conditions will be clearly documented. One virus film will be prepared for each batch of test substance and virus control, per exposure time requested, for subsmission to the EPA or TGA and five virus films will be prepared for each batch of test substance and virus control, per exposure time requested, for subsmission to Health Canada.

TEST METHOD

Preparation of Sephadex Gel Filtration Columns

To reduce the cytotoxic level of the virus-test substance mixture prior to assay of virus, and/or to reduce the virucidal level of the test substance, virus is separated from the test substance by filtration through Sephadex gel. The Sephadex gel is prepared by equilibration with phosphate buffered saline containing 1% albumin. The type of Sephadex used will be specified in the final report. On the day of testing, Sephadex columns are prepared by centrifuging the prepared Sephadex gel in sterile syringes for three minutes to clear the void volume. The columns are now ready to be used in the assay.

Input Virus Control

On the day of testing, the stock virus utilized in the assay will be titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. The results of this control are for informational purposes only.

Treatment of Virus Films with the Test Substance

For each batch of test substance, the required number of dried virus films are exposed to 2.0 mL of the use dilution of the test substance (liquid products), or to the amount of spray released under use conditions (spray products) and held covered for the specified exposure time(s) and temperature. The actual temperature will be recorded. Following the exposure time, each plate is scraped with a cell scraper to resuspend the contents of the plate and the virus-test substance mixture is immediately passed through an individual Sephadex column utilizing the syringe plunger in order to detoxify the mixture. Each filtrate (10^{-1} dilution) is then titered by serial dilution and assayed for infectivity and/or cytotoxicity. To further aid in the removing of the cytotoxic effects of the test substance to the indicator cell cultures, individual dilutions may be passed through a second individual Sephadex column.

Treatment of Virus Control Film

The required number of virus films are prepared as described above for each exposure time assayed. The virus control films are run in parallel to the test virus but 2.0 mL of test medium is added in lieu of the test substance. Each virus control is held covered for the same exposure time and at the same exposure temperature as the test substance. Following the exposure time, the virus film(s) is scraped as previously described and each mixture is immediately passed through an individual Sephadex column utilizing the syringe plunger. Each filtrate (10^{-1} dilution) is then titered by serial dilution and assayed for infectivity. If additional Sephadex columns were used to further reduce the cytotoxic effects in the test substance assay, the same dilutions of the virus control will be passed through individual Sephadex columns.

Cytotoxicity Controls

A 2.0 mL aliquot of each batch of test substance (liquid products) or the amount of the test substance recovered when sprayed onto a sterile petri dish (spray products), is filtered through a Sephadex column utilizing the syringe plunger and the filtrate is diluted serially in medium and inoculated into cell cultures for assay of cytotoxicity concurrently with the virus control and test substance-treated virus samples. For spray products, the cytotoxicity control will be held covered for the longest requested exposure time at the requested exposure temperature. If additional Sephadex columns were used to further reduce the cytotoxic effects in the test substance assay, the same dilutions of the cytotoxicity control will be passed through individual Sephadex columns.

Assay of Non-Virucidal Level of Test Substance
Each dilution of the neutralized test substance (cytotoxicity control dilutions) will be challenged with an aliquot of low titer stock virus to determine the dilution(s) of test substance at which virucidal activity, if any, is retained. Dilutions that show virucidal activity will not be considered in determining reduction of the virus by the test substance.

Using the cytotoxicity control dilutions prepared above, an additional set of indicator cell cultures will be inoculated with a 250 µL aliquot of each dilution in quadruplicate. A 0.1 mL aliquot of low titer stock virus will be inoculated into each cell culture well and the indicator cell cultures will be incubated along with the test and virus control plates.

Infectivity Assays
The RAW 264.7 macrophage cell line will be used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes will be inoculated in quadruplicate with a 250 µL aliquot of the dilutions prepared from the test and control groups. Uninfected indicator cell cultures (cell controls) will be inoculated with test medium alone. The inoculum is allowed to adsorb for approximately 60 minutes at room temperature. Following the adsorption time, the inoculum is removed and an aliquot of MNV Overlay Agarose I is inoculated into each well of the cell culture plates. The cultures are incubated at 36-38°C in a humidified atmosphere of 5-7% CO_2 for approximately two days. Following incubation, an aliquot of MNV Overlay Agarose II containing neutral red stain is added and the cultures are incubated at 36-38°C in a humidified atmosphere of 5-7% CO_2 for approximately four hours. Following incubation, the cell cultures are visually observed for viral specific plaques. The cultures may be microscopically observed to verify plaques or test substance cytotoxicity.

CALCULATION OF TITERS

Viral and cytotoxicity titers will be expressed as $-\log_{10}$ of the 50 percent titration endpoint for infectivity (PFU_{50}) as calculated by the method of Spearman Karber.

$$-\text{Log of 1st dilution inoculated} - \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100}\right) - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Calculation of Log Reduction (EPA and TGA)

Dried Virus Control PFU_{50} – Test Substance PFU_{50} = Log Reduction

Calculation of Log Reduction (Health Canada)

Average Dried Virus Control PFU_{50} – Test Substance PFU_{50} = Log Reduction

PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM

The specialized virucidal testing section of ATS Labs maintains Standard Operating Procedures (SOPs) relative to virucidal efficacy testing studies. Virucidal efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including virus and cell stocks for purposes of identification, receipt and use of chemical reagents including cell culture medium components, etc. These procedures are designed to document each step of virucidal efficacy testing studies. Appropriate references to medium batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each virucidal efficacy test is assigned a unique Project Number when the Study Director initiates the protocol for the study. This number is used for identification of the test culture plates, etc. during the course of the test. Test culture plates are also labeled with reference to the test virus, experimental start date, and test product. These measures are designed to document the identity of the test system.

METHOD FOR CONTROL OF BIAS: N/A

TEST CRITERIA FOR EPA SUBMISSION
A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control; 2) that when cytotoxicity is evident, at least a 3-log reduction in titer is demonstrated beyond the cytotoxic level; 3) that the cell controls be negative for infectivity. If any of the previous requirements are not met, the test may be repeated under the current protocol number. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

TEST CRITERIA FOR HEALTH CANADA SUBMISSION
A valid test requires 1) at least a 4-log infectivity be recovered from the dried virus control beyond the cytotoxic level of the test substance; 2) that the cell controls be negative for infectivity. If any of the previous requirements are not met, the test may be repeated under the current protocol number. **Note:** An efficacious product must demonstrate at least a 3 $\log_{10}$ reduction in viral titer beyond the cytotoxic level of the test substance.

TEST CRITERIA FOR AUSTRALIAN TGA SUBMISSION
A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control; 2) that when cytotoxicity is evident, at least a 3-log reduction in titer is demonstrated beyond the cytotoxic level; 3) that the cell controls be negative for infectivity. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

FINAL REPORT
The report will include, but not be limited to, identification of the sample and date received, dates on which the test was initiated and completed, identification of the virus strain used and composition of the inoculum, description of cells, medium and reagents, description of the methods employed, tabulated results, calculated titers for infectivity and cytotoxicity, and a conclusion as it relates to the purpose of the test.

PROTOCOL CHANGES
If it becomes necessary to make changes in the approved protocol, the revision and reasons for change will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

PRODUCT DISPOSITION
Test substance retention shall be the responsibility of the Sponsor. Unused test material will be **discarded** following study completion unless otherwise requested.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of the final study report.
7. Study-specific SOP deviations made during the study.

Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study, which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

PROPOSED STATISTICAL METHODS: N/A

REFERENCES

1. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1053 (Current Version).
2. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1482 (Current Version).
3. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November 1982.
4. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.
5. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E. H, Lennette, D.A., and Lennette, E.T. editors. Seventh edition, 1995.
6. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.
7. Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices, CAN/CGSB-2.161-97, August 1997.
8. Guidance Document: Disinfectant Drugs, Health Products and Food Branch, Health Canada, 10/29/2007.
9. Therapeutic Goods Administration. Therapeutic Goods Order No. 54: Standards for Disinfectants and Sterilants. February 1998.
10. Therapeutic Goods Administration. Therapeutic Goods Order No. 54A: Amendment to Standards for Disinfectants and Sterilants (TGO 54). March 1997.
11. Therapeutic Goods Administration (TGA). Draft Guidelines for the Evaluation of Household/Commercial and Hospital Grade Disinfectants. July 2005.
12. Therapeutic Goods Administration (TGA). Guidelines for the Evaluation of Sterilants and Disinfectants. February 1998.
13. Wobus CE, Karst SM, Thackray LB, Chang KO, Sosnovtsev SV, et al. (2004) Replication of *Norovirus* in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol 2(12):e432.

STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Sponsor (Date/Initial): 1/19/11

Test Substance (Name and Batch Number - exactly as it should appear on final report):
Clidox S Base Lot# 1710344 w/Activator Lot 1610343 & Clidox-S Base Lot#1711014 w/Activator Lot# 1611013

Expiration Date: Base Lot# 170344 expires 12/10/2011 ; Base Lot# 1711014 expires 01/14/2012
Activator Lot# 1610343 expires 12/09/2011 ; Activator Lot# 1611013 expires 01/13/2012

Product Description

- ☐ Quaternary ammonia
- ☐ Iodophor
- ☐ Sodium hypochlorite
- ☐ Peracetic acid
- ☐ Peroxide
- ☑ Other Sodium Chlorite

Test Substance Active Concentration (upon submission to ATS Labs): 0.85%

Storage Conditions

- ☑ Room Temperature
- ☐ 2-8°C
- ☐ Other _____

Hazards

- ☐ None known: Use Standard Precautions
- ☑ Material Safety Data Sheet, Attached for each product
- ☐ As Follows: _____

Product Preparation

- ☐ No dilution required, Use as received (RTU)
- ☑ *Dilution(s) to be tested:

**Must wait 15 Minutes after product is mixed!

1:5:1 defined as 1 part base + 5 parts water + 1 part activator**
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)

- ☐ Deionized Water (Filter or Autoclave Sterilized)
- ☑ Tap Water (Filter or Autoclave Sterilized)
- ☐ AOAC Synthetic Hard Water: _____PPM
- ☐ Other _____

wait at least 15 minutes after the product is mixed before using it in testing. Per email dated 1/27/11 JC 1/28/11

Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Virus: Murine Norovirus

Exposure Time: 5 Minutes

Exposure Temperature: ☑ Room temperature (18-22°C) (20-22°C for Health Canada)
☐ Other: _____°C(please specify range)

Directions for application of aerosol/spray products: _____
☑ Check here if spray instructions are not applicable.

Organic Soil Load

- ☑ 1% fetal bovine serum
- ☐ 5% fetal bovine serum
- ☐ Other _____

Intended Location of Regulatory Submission

- ☑ U.S. EPA
- ☐ Health Canada
- ☐ Therapeutic Goods Administration (Australia TGA)
- ☐ Not applicable- For internal/other use only

TEST SUBSTANCE SHIPMENT STATUS

☐ Has been used in one or more previous studies at ATS Labs.
☐ Has been shipped to ATS Labs (but has not been used in a previous study).
Date shipped to ATS Labs:_____ Sent via *overnight* delivery? ☐ Yes ☐ No
☑ Will be shipped to ATS Labs.
Date of expected receipt at ATS Labs: 1/20/11
☐ Sender (if other than Sponsor):_____

COMPLIANCE

This study will be conducted in compliance with the EPA Good Laboratory Practices Regulations of 40 CFR Part 160 (Federal Register Notice [August 17, 1989]) and in accordance to standard operating procedures.
☑ Yes
☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

☐ Approved without modification
☑ Approved with modification - Supplemental Information Form Attached - ☐ Yes ☑ No

The input virus control will be inoculated in duplicate. The 10^{-1} dilution for the cytotoxicity and neutralization controls may be incoculated in duplicate or triplicate, if insufficient volume for quadruplicate. The cytotoxicity control will be reported as TCD_{50}.

APPROVAL SIGNATURES

SPONSOR:

NAME: Mr. Jerry Shapiro TITLE: TREASURER

SIGNATURE: [signature] DATE: 1/18/11

PHONE: (800) 243 - 5350 FAX: (203) 755 - 4309 EMAIL: jshapiro@pharmacal.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

ATS Labs:

NAME: Sharen Conway
Study Director

SIGNATURE: Sharen L. Conway
Study Director DATE: 1/28/11